THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 40296–40301, 2002

Printed in U.S.A.

Conformational Changes of Calpain from Human Erythrocytes in the Presence of Ca^{2+}*

Received for publication, May 7, 2002, and in revised form, August 19, 2002
Published, JBC Papers in Press, August 19, 2002, DOI 10.1074/jbc.M204471200

Enrico Dainese‡§, Roberto Minafra¶, Annalaura Sabatucci‡, Patrice Vachette‖, Edon Melloni¶, and Ivo Cozzani‡

From the ‡Department of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, 64100 Teramo, Italy, the ¶Department of Experimental Medicine and Centre of Excellence for Biomedical Research, University of Genoa, Viale Benedetto XV 1, 16032, Genoa, Italy, and ‖Laboratoire pour l'Utilisation du Rayonnement Électromagnétique, Bât. 209d, University of Paris-Sud, B. P. 34, F91898 Orsay Cedex, France

Small angle x-ray scattering has been used to monitor calpain structural transitions during the activation process triggered by Ca^{2+} binding. The scattering pattern of the unliganded enzyme in solution does not display any significant difference with that calculated from the crystal structure. The addition of Ca^{2+} promotes the formation of large aggregates, indicating the exposure of hydrophobic patches on the surface of the protease. In contrast, Ca^{2+} addition in the presence of the thiol proteinase inhibitor E64 or of the inhibitor leupeptin causes a small conformational change with no dissociation of the heterodimer. The resulting conformation appears to be slightly more extended than the unliganded form. From the comparison between *ab initio* models derived from our data with the crystal structure, the major observable conformational change appears to be localized at level of the L-subunit and in particular seems to confirm the mutual movement already observed by the crystallographic analysis of the dII (dIIb) and the dI (dIIa) domains creating a functional active site. This work not only provides another piece of supporting evidence for the calpain conformational change in the presence of Ca^{2+}, but actually constitutes the first experimental observation of this change for intact heterodimeric calpain in solution.

Calpains (EC 3.4.22.17) are a family of cytosolic Ca^{2+}-dependent cysteine endopeptidases widely distributed in all mammalian cells and comprising several genetically distinct isozymes and various homologues in different lower organisms (1, 2). The physiological role of calpains is related to the transduction of extracellular signals mediated by changes in the permeability of membranes to Ca^{2+} or by the mobilization of this ion from internal stores. These proteins are involved in physiological and pathophysiological conditions such as cell cycle regulation, apoptosis, cytoskeletal remodeling, Alzheimer's and Parkinson's diseases, and muscular dystrophies (1, 3–8).

Most biochemical and structural studies concern the two ubiquitous enzymes named μ- and m-calpain exerting their *in vitro* catalytic activity at micromolar (10–50 μM) or millimolar (0.2–0.35 mM) Ca^{2+} concentrations, respectively (1). The calpain from the Ca^{2+}-activated proteolytic system of human erythrocytes is a μ-calpain localized in the cell soluble fraction that *in vitro* binds a maximum of eight equivalents of Ca^{2+} with a K_d of ~25 μM (9). *In vivo*, μ-calpain seems to be activated at physiological Ca^{2+} concentrations of 100–300 nM, suggesting the involvement of other activators (10). These isoforms of calpain are heterodimers consisting of an 80 kDa catalytic large L-subunit and a 28 kDa small S-subunit, whose function probably consists in the regulation of the catalytic one (1). On the basis of amino acid homologies, the L-subunit has been described as comprising four domains, namely dI to dIV, while the S-subunit contains dV and dVI domains (11, 12). Two subdomains dIIa and dIIb are distinguished within the dII domain, which shows some similarity to papain, while dIV and dVI are calmodulin-like domains containing EF-hands. A different domain classification, based on the crystal structure of rat calpain, has also been proposed (13). In the latter, the domain dI includes domain I and subdomain dIIa whereas dII corresponds to the sole subdomain dIIb of the previous nomenclature. Here, we use the second, structure-based, nomenclature accompanied by the first one in parentheses to facilitate reference to previous reports.

In the presence of Ca^{2+}, calpain undergoes autoproteolysis (14). This reaction has been demonstrated to be inhibited by several inhibitors including leupeptin and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64). Previous studies indicate that the binding of both inhibitors requires the presence of Ca^{2+} (9, 15–17). The kinetics of Ca^{2+} binding and the onset of proteolytic activity in native calpain and its isolated subunits have been studied, and activity has been shown to appear only after a delay. This delay has been attributed to a Ca^{2+}-induced conformational change making the active site of the enzyme accessible to the substrate (9).

It has been proposed that Ca^{2+} causes the dissociation of the calpain heterodimer into the constitutive L- and S-subunits (16, 18) thereby yielding the active form of the proteinase (19). However, whether the dissociation has to be considered as the early step in the calpain activation process is still under discussion. Indeed, Nakagawa *et al.* (20) suggested that the autolysis of the protein is necessary for the dissociation of m-calpain, whereas Pal *et al.* (21). observed calpain dissociation, although using the inactive C105S-80k/21k m-calpain form.

The crystal structures of Ca^{2+}-free recombinant human and rat m-calpains have recently been determined at 2.3 and 2.6 Å resolution, respectively (12, 13). In the absence of Ca^{2+}, m-calpain displays a flat oval disk-like shape with the papain-like catalytic domains dI and dII (or dIIa and dIIb) located at

* This work was supported by the 5th Framework Program of the European Commission Access to Research Infrastructures, project BD 005-00 (to E. D.) carried out at LURE. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 39-861-266876; Fax: 39-861-412583; E-mail: dainese@unite.it.

one pole and the calmodulin-like domains dIV-dVI at the opposite pole. The analysis of the crystal structure reveals that some residues of the active site, which are crucial to the proteolytic activity of the protein, are not correctly positioned with respect to the substrate binding site in the unliganded state (12), suggesting the requirement for a structural rearrangement leading to the active form. The authors of the human m-calpain crystal structure postulated the existence of an electrostatic switch caused by the binding of Ca^{2+} at the interface between dII (dIIb) and the negatively charged loop of dIII, a proposal supported by recent studies carried out on calpain mutants (22). More recently, Moldoveanu *et al.* (23) determined the crystal structure of a Ca^{2+}-bound construct of μ-calpain comprising only the dI (dIIa) and dII (dIIb) domains. In this so-called minicalpain structure, Ca^{2+} is seen bound to two non-EF-hand sites, one in each domain, thereby inducing a structural rearrangement of the active site leading to a correct positioning of the catalytic triad. Previous crystallographic studies on the Ca^{2+}-free and Ca^{2+}-bound isolated domain VI homodimer detected very small conformational changes at the level of the S-subunit upon Ca^{2+}-binding (24–26). However, direct experimental evidence of the overall structural modifications occurring in the complete calpain in solution during its activation is still lacking.

We have therefore undertaken a study of the effect of Ca^{2+} and inhibitors of calpain on the enzyme conformation using small angle x-ray scattering (SAXS)[1] in solution. This method has been widely used to determine low resolution protein structures and conformational changes and is very sensitive to modifications of the state of aggregation of proteins in solution (27, 28).

Synchrotron radiation x-ray small angle scattering measurements were carried out on calpain isolated from human erythrocytes. Different solutions of the proteinase were analyzed both in the absence and in the presence of Ca^{2+}. We also investigated the calpain structure in the presence of two different inhibitors, the synthetic inhibitor *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64), which is known to react with the -SH group at the active site, and the neutral serine and thiol protease inhibitor leupeptin, again in the absence or presence of Ca^{2+}. The shape of the unliganded molecule fits well with the envelope of the crystal structure. While Ca^{2+} triggers the aggregation of calpain, the molecule remains heterodimeric in solution in the additional presence of inhibitors, allowing us to detect and characterize a conformational change, which appears to be compatible with the mechanism put forward on the basis of the crystal structures.

EXPERIMENTAL PROCEDURES

Purification of Calpain from Human Erythrocytes—Calpain was purified from human erythrocytes according to Michetti *et al.* (29) and dissolved in 50 mM sodium borate buffer, pH 7.5 containing 0.1 mM EDTA. Protein concentration was determined using the Bradford method (30). The effect of Ca^{2+} was studied both in the presence and in the absence of 100 μM E64 and leupeptin using the following five different Ca^{2+} concentrations in excess with respect to the EDTA concentration (*i.e.* free Ca^{2+}): 10, 25, 100, 200, and 1000 μM.

Scattering Experiments and Data Processing—SAXS measurements were performed using the synchrotron radiation beam line D24 at the DCI storage ring of LURE (Laboratoire pour l'Utilisation du Rayonnement Électromagnétique, Orsay, France). The instrument, the data acquisition system (31), and the evacuated measuring cell (32) have already been described. To avoid any possible protein damage due to x-ray irradiation, the protein solution was slowly circulated in the measuring quartz capillary at the constant temperature of 20 °C. In the course of several measuring sessions, samples from four different preparations were used. Freshly prepared samples of calpain were measured in the buffer used in the final step of purification. The monodispersity of each sample was checked immediately before SAXS measurements by SE-HPLC (Waters 486 System with a Shodex Protein KW-802.5 column). Eight frames of 100 s each were recorded using a position-sensitive proportional detector placed 1819 mm downstream from the sample so as to cover the range of momentum transfer q from 0.01 to 0.15 $Å^{-1}$ ($q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle, λ is the radiation wavelength, λ = 1.488 Å, absorption K-edge of Nickel). Frames were visually inspected to check for x-ray damage; none was found. Data were scaled to the transmitted intensity before computing the average and RMSD of each measurement and subtracting the scattering from the corresponding buffer.

The structural parameters characterizing calpain in solution were calculated using the following standard procedures. At very small angles, the intensity scattered by a particle can be approximated by a Gaussian curve (33) as in Equation 1,

$$I(q) \approx I(0)\cdot\exp(-q^2R_g^2/3) \qquad \text{(Eq. 1)}$$

and the slope of a Guinier plot ($\ln[I(q)]$ *versus* q^2) readily yields the value of the radius of gyration R_g of the particle and the value of the intensity at the origin $I(0)$. This approximation is valid over a restricted q-range (typically $R_gq<1.3$). When put on an absolute scale, *e.g.* by means of a reference sample, $I(0)/c$ (c, protein concentration of the sample) is proportional to the molecular mass m of the protein. Here, the calibration was performed using both the isolated subunit of the hemocyanin from *Carcinus aestuarii* (sedimentation coefficient 5 S, m = 75 kDa) and its hexameric form (sedimentation coefficient 16 S, m = 450 kDa) (34).

The distance distribution function $p(r)$ corresponds to the distribution of distances between any two volume elements within one particle. It has been determined using the indirect transform method as implemented in the program GNOM (35). This function provides an alternative estimate of the radius of gyration derived through the relationship shown in Equation 2.

$$R_g^2 = \frac{\int r^2p(r)dr}{2\int p(r)dr} \qquad \text{(Eq. 2)}$$

Scattering intensities were computed from the atomic coordinates of the crystal structure of human m-calpain (1kfu.pdb) by using the program CRYSOL, which takes into account the hydration water by introducing a 3 Å thick border layer surrounding the molecule (36). The calculated scattering profile is fitted to the experimental pattern using only two adjustable parameters, the excluded volume of the particle V and the electron density in the border layer ρ_b, to minimize the discrepancy as shown in Equation 3,

$$\chi^2 = \frac{1}{N-1}\sum_{i=1}^{N}\left(\frac{I_e(q_i) - I(q_i)}{\sigma(q_i)}\right)^2 \qquad \text{(Eq. 3)}$$

where N is the number of experimental points and $I_e(q_i)$ and $\sigma(q_i)$ denote the experimental scattering curve and its RMSD, respectively.

The *ab initio* shape determination was performed with the dummy atom model (DAM) method (37) using the program DAMMIN running on a Silicon Graphics O2 work station. A sphere of diameter D_{max} is filled by closely packed small spheres (dummy atoms) of radius $r_0 \ll D_{max}$. The DAM structure is defined by a configuration vector $\mathbf{X}$ with $N \approx (D_{max}/r_0)^3$ components. Using simulated annealing, the program searches for a configuration that fits the experimental data while a looseness penalty ensures the compactness and connectivity of the solution (37). No particular condition of oblateness of the particle shape was imposed as constraint in these calculations. The resulting shape together with the high resolution crystal structure were displayed and manipulated using the graphical software package ASSA (38). The superimposition of the pdb structures with the three-dimensional models of the protein obtained with the DAM method was performed using the program SUPCOMB (39).

[1] The abbreviations used are: SAXS, small angle x-ray scattering; E64, *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane; SE-HPLC, size exclusion high pressure liquid chromatography; RMSD, root mean-squared deviation.

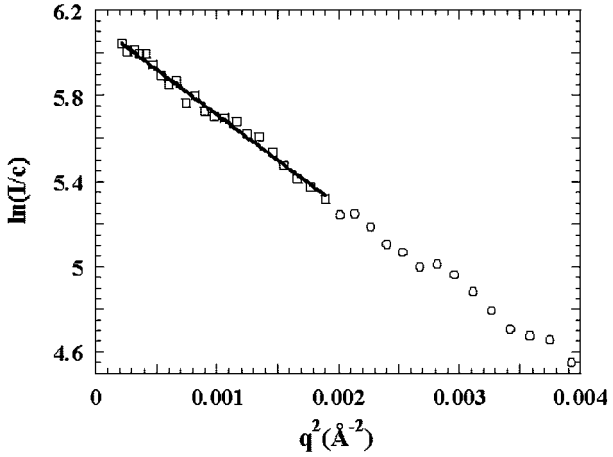

FIG. 1. **Guinier plot of native calpain from human erythrocyte in sodium borate 50 mM buffer, pH 7.5, containing 0.1 mM EDTA at a concentration of 1.6 mg/ml.** The *squares* represent the experimental points used in the linear regression.

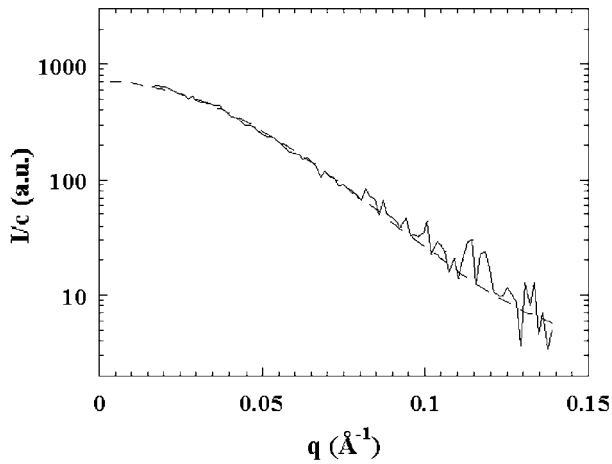

FIG. 2. **Comparison of the experimental scattering intensity of unliganded calpain from human erythrocytes in solution (*solid line*) with the pattern calculated from the atomic coordinates of human m-calpain (1kfu.pdb) using the program CRYSOL (*dashed line*).**

RESULTS AND DISCUSSION

Structural Characterization of Unliganded Calpain in Solution—The unliganded μ-calpain, at a concentration of 1.6 mg/ml (14.8 μM), shows a value of the radius of gyration calculated from the Guinier analysis of the scattering intensity (Fig. 1) of R_g = 35.8 ± 0.4 Å. The molecular mass of the native protein calculated from the zero-angle intensity has a value of 110 ± 10 kDa, in close agreement with the value of 108 kDa typical for both μ- and m-isoforms of calpain (1). This confirms that under our experimental conditions protein solutions are monodisperse (*i.e.* no species with a different molecular mass can be detected) and the interparticle interactions are negligible. Calculation of the pair distribution function of the unliganded enzyme yields a value for the maximal diameter D_{max} of the protein of 120 Å with a value of the radius of gyration of 36.3 ± 0.4 Å, very close to that derived from the Guinier analysis.

The program CRYSOL was used to calculate the SAXS pattern from the atomic coordinates of Ca^{2+}-free human m-calpain (1kfu.pdb), which is shown in Fig. 2 together with the experimental scattering curve. The calculated curve, which corresponds to a dry excluded volume of 122 nm^3 and to an electron density contrast in the solvation shell $\delta\rho_b$ = 44 e/nm^3 (*i.e.* density of the solvent in the shell 1.13 g/cm^3), neatly fits our experimental data (χ = 1.395). In parallel, an *ab initio* determination of the overall shape of the unliganded μ-calpain from the SAXS pattern was performed using the program DAMMIN (see "Experimental Procedures"). Ten independent calculations were performed and yielded very similar shapes. A typical result is presented in Fig. 3 superimposed on the crystal structure of the human m-calpain. The shape provides a satisfactory fit envelope to the crystal structure.

The observations regarding the scattering patterns and the models indicate that the overall organization of both isoforms of the proteinase are very similar. This is not unexpected, considering that the high resolution structure of m-calpain from rat[2] (13) is very similar to its human counterpart[3] (12) with an RMSD over 800 C_α atoms of 1.55 Å. It also demonstrates that the overall shape of the protein does not undergo any significant distortion inside the crystal lattice, a non-trivial result in the case of a multidomain protein for which significant differences between the structure in solution and in the crystal may be observed (40).

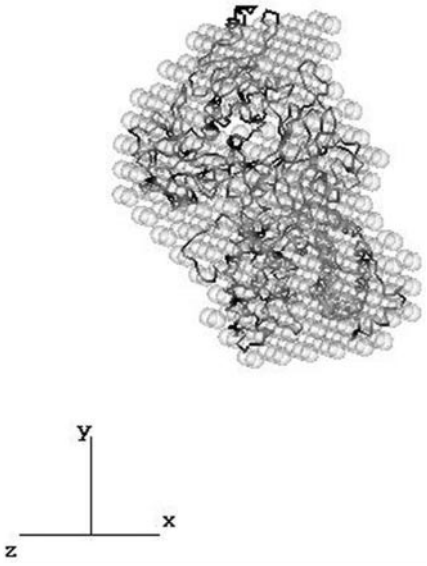

FIG. 3. **Superimposition of the low-resolution shape of Ca^{2+}-free human calpain.** This was obtained with the program DAMMIN (*gray spheres*) with the crystal structure 1kfu.pdb (C chain) performed with the program SUPCOMB (see "Experimental Procedures").

Effect of Ca^{2+} Addition—μ-Calpain solutions were studied in the presence of variable amounts of Ca^{2+}. At 10 and 25 μM free Ca^{2+}, the scattering curves were identical to that obtained in the absence of Ca^{2+} (data not shown). In the presence of 100 μM (and 200 μM) free Ca^{2+}, the SAXS pattern displays conspicuous differences from that of the unliganded protease (Fig. 4, *dashed line*), most notably a marked upward curvature at small angles, which is an unambiguous indication of the formation of large soluble aggregates with a broad size distribution. The addition of 1 mM free Ca^{2+} causes the formation of amorphous precipitates. Furthermore, preliminary experiments show that the aggregation process observed at 100 μM Ca^{2+} is essentially reversed upon addition of an excess of EDTA or in the presence of 100 mM NaCl, KCl, or NaSCN, confirming the observations of Pal *et al.* (21).

[2] Research Collaboratory for Structural Bioinformatics Protein Databank, 1df0.pdb. The atomic coordinates for the crystal structure of this protein are available in the Molecular Modeling Database (www.nnlm.nlm.nih.gov/mar/molbio/3D/index.htm) under MMDB: 13622 (13).

[3] Research Collaboratory for Structural Bioinformatics Protein Databank, 1kfu.pdb. The atomic coordinates for the crystal structure of this protein are available in the Molecular Modeling Database (www.nnlm.nlm.nih.gov/mar/molbio/3D/index.htm) under MMDB: 18112 (12).

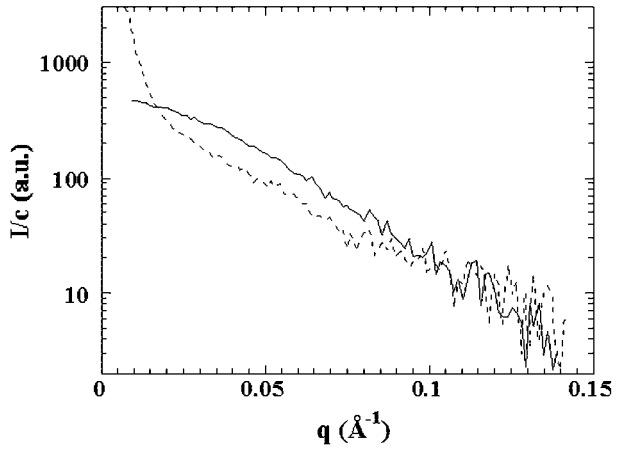

FIG. 4. **Comparison between the SAXS patterns of native calpain in the absence (*solid line*) and in the presence (*dashed line*) of 100 μM free Ca^{2+}.**

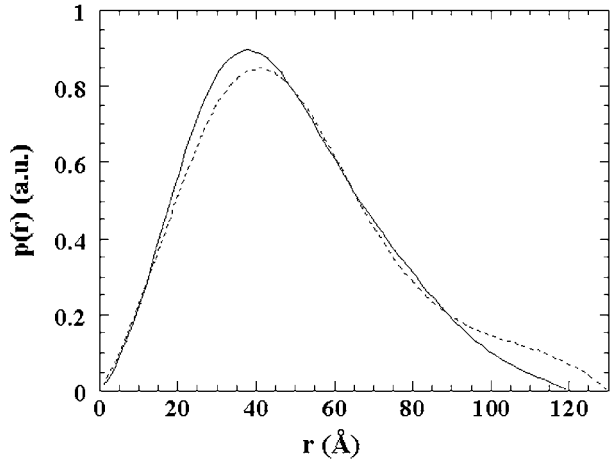

FIG. 5. **Comparison of the $p(r)$ function of unliganded calpain (*solid line*) with the protein after the addition of 100 μM E64 in the presence of 100 μM free Ca^{2+} (*dashed line*).**

Conformational Change upon Ca^{2+} Addition in the Presence of Inhibitors—Although aggregation is thought to originate from a conformational change triggered by Ca^{2+}, its effect dominates the scattering pattern at small angles to such an extent that this putative modification in shape cannot even be detected. To investigate whether the aggregation phenomenon can be related to the autoproteolysis process, we resorted to inhibitors of calpain. E64 is a specific inhibitor for cysteine proteases, which irreversibly binds to the SH group at the active site forming a thioether linkage. In the case of calpains, this inhibitor is effective only in the presence of Ca^{2+} (9, 17), as is the competitive inhibitor leupeptin (15).

In the presence of either E64 or leupeptin inhibitor at a concentration of 100 μM, the addition of 100 or 200 μM free Ca^{2+} does not cause the previously observed strong increase in the intensity at small angles. This means that no high molecular mass aggregates are formed. The Guinier analysis of each scattering curve yields a slightly larger value of the radius of gyration (R_g = 38.5 ± 0.7 Å) with respect to the value calculated for the protein in the absence of Ca^{2+}. Furthermore, the comparison of the pair distribution functions of the protein under these two experimental conditions (Fig. 5) shows a shift to higher distances in the position of the maximum of the $p(r)$ function (r_{max}) by about 4 Å, while the maximum dimension of the particle (D_{max}) increases from 120 to 130 Å in the presence of Ca^{2+}. The value of $I(0)/c$ (where c is the protein concentration), and therefore the molecular mass of the protein, remains constant, which rules out any significant dissociation of calpain into its subunits. The absence of large aggregates suggests that the observed small conformational change induced by Ca^{2+} binding in the presence of inhibitors, does not lead to the exposure of hydrophobic surfaces. In contrast, the addition of 1 mM Ca^{2+} leads to the formation of amorphous precipitates, as already observed in the absence of inhibitor. Thus, in the presence of inhibitor, the solution of calpain remains monodisperse upon the addition of 100 or 200 μM free Ca^{2+}; the protein does not dissociate either but undergoes a transition to a more extended conformation. As a control that this change in the scattering pattern was not due to a spurious effect of the inhibitor, scattering curves were recorded with solutions of human erythrocyte calpain containing 100 μM of each inhibitor but no free Ca^{2+}. The two curves are virtually indistinguishable from that of the unliganded molecule (data not shown).

The calpain aggregation process together with subunit dissociation have been systematically studied with the inactive C105S-80k/21k m-calpain from rat using light scattering (21).

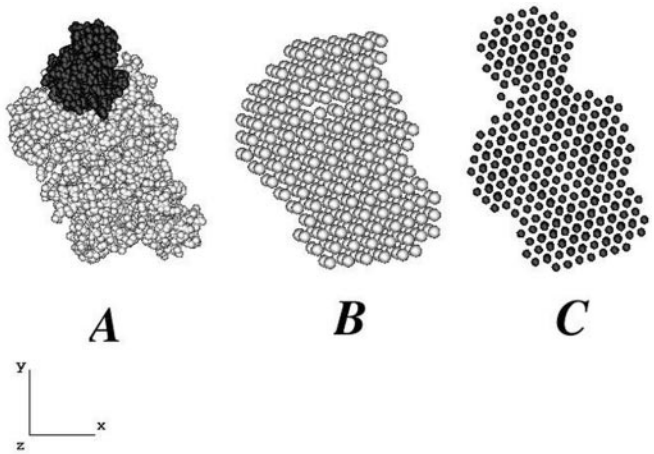

FIG. 6. *A*, crystal structure of unliganded calpain (1kfu.pdb); the *darker region* corresponds to the dII (dIIa-dIIb) domain; *B*, typical shape determined *ab initio* for unliganded calpain; *C*, typical shape determined *ab initio* for human calpain in the presence of 100 μM free Ca^{2+} and 100 μM E64.

In particular, using both Tris and HEPES buffers at a pH value around neutrality and with a protein concentration of 0.7 μM, the Ca^{2+} concentration required for inactive C105S-80k/21k m-calpain to aggregate ranges from 0.8 to 2 mM. These values are well in excess of the free Ca^{2+} concentration of only 100 μM at which we reproducibly observed the formation of large soluble aggregates, but the concentration of μ-calpain used in our SAXS measurements was 14.6 μM (*i.e.* more than 20-fold that used in light scattering measurements). Since the subunit dissociation is a process dependent on protein concentration, ionic strength and salt concentration, a direct and detailed comparison between our results and the studies of Pal *et al.* (21) is hindered by the differences between proteins (*i.e.* human μ-calpain *versus* rat m-calpain), by the large differences in the concentrations used, and by the different sensitivity of the various methods to the presence of small amounts of oligomers. In the conclusion to the study by Pal *et al.* (21), the subunit dissociation is described as a dynamic process triggered by Ca^{2+}, and the aggregation is said to be most likely caused by the exposure of hydrophobic patches from the dimerization surfaces following dissociation.

Based on this view, a possible explanation for our observations suggests itself: the persistence of the heterodimeric form in the presence of Ca^{2+} and inhibitors might reflect the influ-

ence of inhibitors on the dynamic equilibrium of subunit association/dissociation triggered by Ca^{2+}, which could be shifted toward association because of structural constraints imposed by inhibitor binding. However, while this proposal could apply in the case of the larger natural inhibitor calpastatin, which binds to both subunits and thereby stabilizes the heterodimer (21, 41), it seems of little relevance in the case of the binding of the small inhibitors used in our work. Indeed, the crystal structures of complexes of papain with E64 (42) and leupeptin (43) show both inhibitors inserted in the active site, far from the interface between L- and S-subunits. Assuming that inhibitors bind in a similar way to calpain, their influence on the subunit association could only be indirect and most likely limited.

An alternative explanation for the persistence of the heterodimeric form in the presence of Ca^{2+} and inhibitors is that under our experimental conditions (*i.e.* Ca^{2+}/calpain molar ratio of ~7–14), Ca^{2+} binding causes the small conformational change observed by SAXS with no detectable effect on the dynamic dissociation/association equilibrium and that the alteration of the latter can only occur at higher Ca^{2+} concentration (precipitates are observed at 1 mM also with inhibitors) or following autoproteolysis in the absence of inhibitors. It seems plausible that the conformational change observed by SAXS in the μ-calpain from human erythrocytes in the presence of inhibitors and 100 μM Ca^{2+} also occurs in the case of the inactive C105S-80k/21k m-calpain but at a Ca^{2+} concentration lower than that required for aggregation as determined by light scattering (21). This proposal could be established using a technique sensitive to conformation changes such as SAXS. Nevertheless, our results are already supported by the observation that the inactive mutant form of m-calpain C105S-m80k/30k does not dissociate in the presence of Ca^{2+} as recently reported by Nakagawa *et al.* (20).

The results of ten independent *ab initio* analyses, carried out using the program DAMMIN on the SAXS data of the proteinase in the presence of 100 μM E64 and 100 μM free Ca^{2+}, yield similar low resolution models. A typical model is reported in Fig. 6, *model C*. As already suggested by the observed increase of both the r_{max} and the D_{max} values from the $p(r)$ analysis, the resulting shape appears to be more elongated than the model obtained for the native calpain (Fig. 6, *model B*). Most noticeable is the appearance of a protuberance at one end of the particle separated from the rest of the molecule by a slight constriction like a neck barely visible or absent on the models for the unliganded enzyme.

The crystal structure of the unliganded enzyme shows that the two domains dI and dII (or dIIa and dIIb) contain the catalytic triad responsible for the protease activity and are located at one end of the molecule (12, 13). The active site is however split in two parts, one on each domain, and a mutual movement bringing the two domains together is required to activate the enzyme, an event triggered by Ca^{2+} binding. Using a μI-II minicalpain, which could be crystallized in the presence of Ca^{2+}, a conformational change has been recently observed that brings the two domains dI (dIIa) and dII (dIIb) together and the active site residues in a suitable position for catalysis (23). When we consider this process in the context of the entire heterodimer, a more compact protuberance would be formed at the upper end of the proteinase, in agreement with our results. Moreover, at the opposite end of the protein our *ab initio* model of the Ca^{2+}-bound calpain does not display significant differences with the model obtained for the unliganded form (Fig. 6, *C versus B* and *A*). These results are in agreement with the crystallographic studies carried out on the Ca^{2+}-free and Ca^{2+}-bound isolated domain VI homodimer of the S-subunit that detected very localized conformational changes in this region of the proteinase (25), which would go unnoticed using SAXS (24, 26).

In conclusion, our results constitute the first direct experimental evidence that Ca^{2+} binding, in the presence of inhibitor, triggers a conformational transition of the undissociated heterodimeric form of calpain. Although at low resolution, our model is compatible with the already observed dI-dII (dIIa-dIIb) movement (23). Indeed, inhibitors which cannot bind in the absence of Ca^{2+} do so irreversibly with high reactivity (E64) (9) or with high affinity (leupeptin, μ-calpain $K_i = 3.2 \times 10^{-7}$ M, m-calpain $K_i = 4.3 \times 10^{-7}$ M) (15) in the presence of Ca^{2+}, which could imply that the active site undergoes a conformational change leading to the active conformation as already suggested by Crawford *et al.* (17). This result, together with previous studies discussed above, suggests that in the presence of Ca^{2+} and inhibitors, human μ-calpain could also adopt an active conformation without subunit dissociation. Clearly, this proposal can only be validated by the high resolution structure of our complex. Precisely, the availability of monodisperse solutions of Ca^{2+}-bound μ-calpain open the way to the crystallization and subsequent high resolution structural analysis of the intact heterodimeric calpain in the presence of Ca^{2+}. The conformational change observed in the presence of Ca^{2+} and inhibitors could be a likely requirement for the interaction with the natural inhibitor, calpastatin (14, 44), to which we are currently extending our studies.

Acknowledgment—We thank the technical staff of LURE-DCI.

REFERENCES

1. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) *Biochem. J.* **328,** 721–732
2. Sorimachi, H., and Suzuki, K. (2001) *J. Biochem.* (*Tokyo*) **129,** 653–664
3. Spencer, M. J., Croall, D. E., and Tidball, J. G. (1995) *J. Biol. Chem.* **270,** 10909–10914
4. Carafoli, E., and Molinari, M. (1998) *Biochem. Biophys. Res. Commun.* **247,** 193–203
5. Richard, I., Roudaut, C., Saenz, A., Pogue, R., Grimbergen, J. E., Anderson, L. V., Beley, C., Cobo, A. M., de Diego, C., Eymard, B., Gallano, P., Ginjaar, H. B., Lasa, A., Pollitt, C., Topaloglu, H., Urtizberea, J. A., de Visser, M., van der, K. A., Bushby, K., Bakker, E., Lopez, d. M., Fardeau, M., and Beckmann, J. S. (1999) *Am. J. Hum. Genet.* **64,** 1524–1540
6. Santella, L., Kyozuka, K., Hoving, S., Munchbach, M., Quadroni, M., Dainese, P., Zamparelli, C., James, P., and Carafoli, E. (2000) *Exp. Cell Res.* **259,** 117–126
7. Xu, Y., and Mellgren, R. L. (2002) *J. Biol. Chem.* **277,** 21474–21479
8. Kulkarni, S., Goll, D. E., and Fox, J. E. (2002) *J. Biol. Chem.* **277,** 24435–24441
9. Michetti, M., Salamino, F., Minafra, R., Melloni, E., and Pontremoli, S. (1997) *Biochem. J.* **325,** 721–726
10. Melloni, E., Michetti, M., Salamino, F., and Pontremoli, S. (1998) *J. Biol. Chem.* **273,** 12827–12831
11. Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., and Suzuki, K. (1984) *Nature* **312,** 566–570
12. Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K., and Bode, W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 588–592
13. Hosfield, C. M., Elce, J. S., Davies, P. L., and Jia, Z. (1999) *EMBO J.* **18,** 6880–6889
14. Melloni, E., Michetti, M., Salamino, F., Minafra, R., and Pontremoli, S. (1996) *Biochem. Biophys. Res. Commun.* **229,** 193–197
15. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) *J. Biol. Chem.* **259,** 12489–12494
16. Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) *FEBS Lett.* **358,** 101–103
17. Crawford, C., Brown, N. R., and Willis, A. C. (1993) *Biochem. J.* **296,** 135–142
18. Suo, S., Koike, H., Sorimachi, H., Ishiura, S., and Suzuki, K. (1999) *Biochem. Biophys. Res. Commun.* **257,** 63–66
19. Bessiere, P., Cottin, P., Balny, C., Ducastaing, A., and Bancel, F. (1999) *Biochim. Biophys. Acta* **1430,** 254–261
20. Nakagawa, K., Masumoto, H., Sorimachi, H., and Suzuki, K. (2001) *J. Biochem.* (*Tokyo*) **130,** 605–611
21. Pal, G. P., Elce, J. S., and Jia, Z. (2001) *J. Biol. Chem.* **276,** 47233–47238
22. Hosfield, C. M., Moldoveanu, T., Davies, P. L., Elce, J. S., and Jia, Z. (2001) *J. Biol. Chem.* **276,** 7404–7407
23. Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z., and Davies, P. L. (2002) *Cell* **108,** 649–660
24. Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S., Davies, P. L., Elce, J. S., and Cygler, M. (1997) *Nat. Struct. Biol.* **4,** 532–538
25. Lin, G. D., Chattopadhyay, D., Maki, M., Wang, K. K., Carson, M., Jin, L., Yuen, P. W., Takano, E., Hatanaka, M., DeLucas, L. J., and Narayana, S. V. (1997) *Nat. Struct. Biol.* **4,** 539–547

26. Cygler, M., Grochulski, P., and Blanchard, H. (2002) *Methods Mol. Biol.* **172,** 243–260
27. Grossmann, J. G., Sharff, A. J., O'Hare, P., and Luisi, B. (2001) *Biochemistry* **40,** 6267–6274
28. Shi, L., Kataoka, M., and Fink, A. L. (1996) *Biochemistry* **35,** 3297–3308
29. Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E., and Pontremoli, S. (1996) *FEBS Lett.* **392,** 11–15
30. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254
31. Boulin, C., Kempf, R., Koch, M. H. J., and McLaughlin, S. M. (1986) *Nucl. Instrum. and Meth.* 399–407
32. Dubuisson, J. M., Decamps, T., and Vachette, P. (1997) *J. Appl. Cryst.* **30,** 49–54
33. Guinier, A., and Fournet, G. (1955) *Small Angle scattering of X-rays*, Wiley, New York
34. Dainese, E., Di Muro, P., Beltramini, M., Salvato, B., and Decker, H. (1998) *Eur. J. Biochem.* **256,** 350–358
35. Svergun, D. I. (1992) *J. Appl. Crystallogr.* **25,** 495–503
36. Svergun, D. I., Barberato, C., and Koch, M. H. (1995) *J. Appl. Crystallogr.* **28,** 768–773
37. Svergun, D. I. (1999) *Biophys. J.* **76,** 2879–2886
38. Kozin, M. B., Volkov, V. V., and Svergun, D. I. (1997) *J. Appl. Crystallogr.* **30,** 811–815
39. Kozin, M. B., and Svergun, D. I. (2001) *J. Appl. Crystallogr.* **34,** 33–41
40. Svergun, D. I., Barberato, C., Koch, M. H., Fetler, L., and Vachette, P. (1997) *Proteins* **27,** 110–117
41. Takano, E., Ma, H., Yang, H. Q., Maki, M., and Hatanaka, M. (1995) *FEBS Lett.* **362,** 93–97
42. Varughese, K. I., Ahmed, F. R., Carey, P. R., Hasnain, S., Huber, C. P., and Storer, A. C. (1989) *Biochemistry* **28,** 1330–1332
43. Schroder, E., Phillips, C., Garman, E., Harlos, K., and Crawford, C. (1993) *FEBS Lett.* **315,** 38–42
44. Pontremoli, R., Melloni, E., and Salamino, F. (1999) in *Calcium as a cellular regulator* (Carafoli, E., and Klee, C., eds) pp. 371–388, Oxford Press, New York-Oxford

Conformational Changes of Calpain from Human Erythrocytes in the Presence of Ca^{2+}

Enrico Dainese, Roberto Minafra, Annalaura Sabatucci, Patrice Vachette, Edon Melloni and Ivo Cozzani

J. Biol. Chem. 2002, 277:40296-40301.
doi: 10.1074/jbc.M204471200 originally published online August 19, 2002
